A novel pair of two-component signal transduction system ecrE₁/ecrE₂ regulating antibiotic biosynthesis in Streptomyces coelicolor

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Abstract: Two-component signal transduction system (TCS) is a common mechanism of signal transduction and exists in almost all bacterial species. The putative two-component genes SCO6421 (ecrE₁) and SCO6422 (ecrE₂) in Streptomyces coelicolor, which located in the vicinity of the red locus, could modulate the antibiotic undecylprodigiosin (Red) biosynthesis. In this study, the mutants of M145E1 (ecrE₁ knock-out) and M145E2 (ecrE₂ knock-out) were constructed using homologous recombination by double crossover. Red production of two mutants decreased dramatically compared with that of the wild-type strain. However, the production of actinorhodin (Act) of two mutants had not changed significantly. The results suggested that ecrE₁/ecrE₂ participated in regulating the antibiotic Red synthesis. Northern blotting analysis indicated that ecrE₁/ecrE₂ may regulate the biosynthesis of Red through influencing the transcription of redD and redZ during cell growth. Therefore, ecrE₁/ecrE₂, as a specific pair of TCS, most likely regulates the red gene cluster positively.

Key words: ecrE₁/ecrE₂; Streptomyces coelicolor; two-component signal transduction system; undecylprodigiosin (Red).

Abbreviations: Act, actinorhodin; ecr, expression coordinated with Red; Red, undecylprodigiosin; TCS, two-component signal transduction system.

Introduction

Streptomyces coelicolor has been extensively studied as a model organism. Its ability to produce four antibiotics named actinorhodin (Act), undecylprodigiosin (Red), calcium-dependent antibiotic and methylenomycin, has contributed to study of the pathway-specific and pleiotropic regulation of antibiotic production (Hopwood et al. 1995). Two-component signal transduction system (TCS) is a common mechanism of signal transduction and exists in almost all bacterial species. The genome of S. coelicolor includes numerous putative TCSs consisting of sensor kinase and response regulator (Hutchings et al. 2004). Among these systems, AbsA₁/AbsA₂, AbsQ₁/AbsQ₂ and CutR/CutS are known to affect antibiotic production in S. coelicolor (Ishizu kaet al. 1992; Chang et al. 1996; Aceti & Champness 1998; Anderson et al. 2001). The pathway-specific regulator genes actII-ORF₄ and redD control the production of two pigmented antibiotics, Act and Red, respectively (Takano et al. 1992; Arias et al. 1999). Several ecr (expression coordinated with Red) ORFs located in the vicinity of the red locus have been found using DNA microarrays. Among these ecr ORFs, ecrA₂/A₁ (SCO2518/SCO2517) and ecrE₁/E₂ (SCO6421/SCO6422) encode respectively the sensor kinase and response regulator of putative TCS (Huang et al. 2001). The ecrA₁/ecrA₂ was confirmed to be a pair of TCS positively regulating the biosynthesis of Red (Li et al. 2004). However, as a putative TCS, the function of ecrE₁/ecrE₂ has not been deciphered yet.

In this study, two mutants (knock-out ecrE₁ and ecrE₂) have therefore been constructed and the results demonstrated that ecrE₁/ecrE₂ is a specific pair of TCS, which positively regulates the red gene cluster.

Material and methods

Bioinformatics analysis

Sequence analysis of the two TCSs of EcrA₁/EcrA₂ and EcrE₁/EcrE₂ were carried out using DNAman software (http://www.lynnon.com/) including amino acid sequences alignment and the homology tree plotting.

Plasmids, strains and culture

In this work, Dr. David H. Sherman (University of Michigan, Ann Arbor, MI, USA) presented the plasmids of the modi-
DNA manipulations were carried out as described by SampKCE2004 (Kieser et al. 2000), and also gifted the wild-type strain S. coelicolor M145 kindly.

_Escherichia coli_ DH5α was used as the host for generating recombinant plasmids, and _E. coli_ ET12567 was used to obtain demethylated plasmids before transformation to _S. coelicolor_ M145 protoplast. These _E. coli_ strains were grown in Luria-Bertani (LB) medium supplemented with appropriate antibiotic such as 50 μg/mL ampicillin, 50 μg/mL kanamycin or 100 μg/mL ampicillin. _S. coelicolor_ wild-type M145 (WT) was grown at 30°C and maintained using standard procedures (Kieser et al. 2000). For broth culture, the spores were pre-germinated in 2×YT medium (in per L including 16 g bacto-tryptone, 10 g bacto-yeast extract and 5g NaCl), then inoculated into modified liquid medium R− (pH 7.2) lacking additional KH2PO4, CaCl2 and L-proline before autoclaving containing per L: 103 g sucrose, 10 g glucose, 5 g bacto-yeast extract, 0.1 g dico casaminoacids, 0.25 g K2SO4, 10.12 g MgCl2.6H2O, 2 mL trace element solution, 5.73 g TES, 60 g PEG 8000 (in liquid fermentative medium) or 22 g agar powder (in solid plate medium). YEME medium was used for regeneration of _S. coelicolor_ protoplast which contains per L: 3.0 g bacto-yeast extract, 5.0 g bacto-trypotene, 10.0 g bacto-malt extract, 10.0 g glucose, 340.0 g sucrose, appended 5% glycine and 2 mL 2.5M MgCl2.6H2O after autoclaving. All culture media used in this research work were prepared with guidance of _Practical Streptomyces genetics_ (Kieser et al. 2000).

**Construction of the recombinant plasmids pKCE2003 and pKCE2004**

DNA manipulations were carried out as described by Sambrook et al. (1989). The plasmid pKCI139 as a host vector was digested with BamHIII/HindIII to construct the recombinant plasmids of pKCE2003 and pKCE2004. For pKCE2003, the insert fragment included the 1.06 kb LA1 (upstream of _ecr_1), 0.99 kb RA1 (downstream of _ecr_1) and kanamycin resistance gene sequence. Similarly, for pKCE2004, the 0.866 kb LA2 (upstream of _ecr_2), the 0.866 kb RA2 (downstream of _ecr_2) and kanamycin resistance gene were contained. The sketch map of construction plasmids pKCE2003 and pKCE2004 are shown in Figure 1A. These homologous fragments were amplified by PCR using genomic DNA of _S. coelicolor_ M145 as template. The kanamycin resistance gene was amplified using modified pUC119 (kanα) as template. All primers used in this work are shown in Table 1.

**Construction and screening of mutants**

The recombinant plasmids pKCE2003 and pKCE2004 were transformed into _E. coli_ ET12567 to obtain the demethylated plasmids before transformed into _S. coelicolor_ M145 protoplasts, respectively. Double crossover recombination with corresponding host genes in _S. coelicolor_ M145 chromosome occurred with homologous sequences inserted in the recombinant plasmids pKCE2003 or pKCE2004 (Fig. 1B). The transformatants were propagated on a non-selection R5 medium at 30°C for 14–20 h, then spread with 2 mL of 100 μg/mL apramycin solution and incubated for 2–3 days. The colonies were transferred on R5 plate supplemented with 50 μg/mL kanamycin.

**PCR and southern blotting analysis**

PCR and southern blotting were applied to confirm the mutants. For mutant of M145E1, primers for PCR were the forward primer of LA1 and the reverse primer of RA1. Similarly, for mutant of M145E2, primers for PCR were the forward primer of LA2 and the reverse primer of RA2. PCR templates were the genomic DNA of the mutant M145E1 and M145E2, respectively. PCR products from the genomic DNA of the wild-type _S. coelicolor_ M145 were used as control.

Furthermore, southern blotting was conducted to confirm the mutants. The genomic DNA of M145E1, M145E2 and wild-type _S. coelicolor_ M145 were digested with _PstI_, separated by agarose gel electrophoresis, and transferred to nylon membranes. The probes of LKR1 and LKR2 labeled with digoxigenin were prepared by PCR using pKCE2003 and pKCE2004 as template, respectively. Primers for LKR1 and LKR2 were the forward primer of LA1 and the reverse primer of RA1, and the forward primer of LA2 and the reverse primer of RA2, respectively. Hybridization was carried out at 65°C for 16 hours and then stringency washes were performed. The labeled DNA was detected by the chemiluminescence procedure (Amersham Biosciences, Boehringer Mannheim, USA).

**Morphological analysis**

For morphological study, the mutants and wild-type strain grew on R5 medium plate for 9 days at 30°C. The characteristics of colony were investigated and imaged.

**Fermentation and assay of antibiotics**

Firstly, the spores were pre-germinated in 2×YT medium at 30°C and 200 rpm for 6–8 h, and then inoculated with volume 10% (v/v) into 100 mL of modified liquid medium R− in 500 mL conical flask at 30°C and 220 rpm for 5 days. PEG 8000 was added to accelerate cell dispersal. Every test was repeated three times, which included five parallel flasks in one time, and the result is the average value of three times.

The assay procedures for antibiotic and growth curves were described as reference (Li et al. 2004). Briefly, for Act,

<table>
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<tr>
<th>Fragment</th>
<th>Primersa</th>
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<tr>
<td>LA1</td>
<td>5′-AAGCAAGCTTGCGTCCGTGATCAGGTCGACGGCGC-3′</td>
<td>HindIII</td>
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<tr>
<td></td>
<td>5′-AAAGGAACTCTATGGCCGACACCTATGGCGTCC-3′</td>
<td>EcoRI</td>
</tr>
<tr>
<td>RA1</td>
<td>5′-AACGCTGAGTCCCTGGGAGGAGCCCAGGTAG-3′</td>
<td>PstI</td>
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<tr>
<td></td>
<td>5′-TATAGGAGTCCGAGGCGGATCCGTCGAGG-3′</td>
<td>BamHI</td>
</tr>
<tr>
<td>LA2</td>
<td>5′-GCAGAGCTTGCGTCCGTGATCAGGTCGACGGCGC-3′</td>
<td>HindIII</td>
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<tr>
<td></td>
<td>5′-AGAAGAACCTCTATGGCCGACACCTATGGCGTCC-3′</td>
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<td>RA2</td>
<td>5′-AAAGGAACTCTATGGCCGACACCTATGGCGTCC-3′</td>
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<tr>
<td></td>
<td>5′-ATTTGAATTCAGAGATTCCCTGGATACCGCTCGC-3′</td>
<td>BamHI</td>
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<tr>
<td>Km</td>
<td>5′-TATTCTGCGAGCTGAGTCCCTGGGAGGAGCCCAGGTAG-3′</td>
<td>EcoRI</td>
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a Forward primer: upper line; reverse primer: below line. The restriction sites are underlined.

### Table 1. Oligonucleotides used as primers to amplify the fragments for construction the mutants.

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the sample was treated with 1 M KOH to pH 12.0, and then the culture was filtered through a 0.22 µm filter. Absorbance of the filtrate was determined at 640 nm. For Red, the sample was first extracted with 1 M KOH to separate Act as above, and then centrifuged at 3000×g for 5 min. The mycelia pellet was washed with distilled water and dried by vacuum, and then extracted with pH 2.0 methanol acidified with 1 M HCl overnight at room temperature. The filtrate was detected at 530 nm after filtering through a 0.22 µm filter.

Transcription analysis
In order to gain insight into the possible role of EcrE\textsubscript{1}/EcrE\textsubscript{2} on regulating antibiotic biosynthesis, transcription analysis of both red\textsubscript{D} (SCO5877) and red\textsubscript{Z} (SCO5881), as well as act\textsubscript{II}-ORF\textsubscript{4} (SCO5085) were done using northern blotting. The total RNA indicated the quantity of RNA and wild-type M145 was used as control. The total RNA of \textit{S. coelicolor} M145 and that of mutants were extracted, and northern hybridization proceeded according to the standard method (Sambrook et al. 1989). Total RNA was separated through 1.5% agarose gel electrophoresis containing 2.2 M formaldehyde, and then transferred to nylon membranes. Prehybridization and hybridization proceeded according to the method of observed divergence. The probes were labeled with digoxigenin-dUTP using the genes of red\textsubscript{D}, red\textsubscript{Z}, and act\textsubscript{II}-ORF\textsubscript{4} as templates, respectively. The hybridized membrane was detected by chemiluminescent imager (Amersham Biosciences, Boehringer Mannheim, USA).

Results
Bioinformatics analysis
The amino acid alignment showed that the identity of EcrA\textsubscript{1} and EcrE\textsubscript{2} is 56.46% (118/209), and the identity of EcrA\textsubscript{2} and EcrE\textsubscript{1} is 33.33% (105/315). Furthermore, as shown in Figure 2, there is an evolutionary distance between both TCSs based on the distance method of observed divergence. Thus the \textit{ecrE\textsubscript{1}}/\textit{ecrE\textsubscript{2}} may be another TCS regulating the production of antibiotic of \textit{S. coelicolor} like that of \textit{ecrA\textsubscript{1}}/\textit{ecrA\textsubscript{2}}, in which \textit{ecrA\textsubscript{2}}/\textit{ecrE\textsubscript{1}} may encode a sensor kinase and \textit{ecrA\textsubscript{1}}/\textit{ecrE\textsubscript{2}} may encode a response regulator.

Construction and confirmation of mutants
The homologous sequences inserted in the recombinant plasmids pKCE2003 or pKCE2004 underwent a double crossover recombination with corresponding host genes in \textit{S. coelicolor} M145 chromosome (Fig. 1B). The positive clones taking double crossover recombination were screened on the R\textsubscript{5} plate with kanamycin, which were denoted as M145E\textsubscript{1} (\textit{ecrE\textsubscript{1}} knock-out) and M145E\textsubscript{2} (\textit{ecrE\textsubscript{2}} knock-out).

The mutants were confirmed by PCR and southern blotting analysis. For mutant of M145E\textsubscript{1}, the amplified DNA fragments are 3.15 kb and 3.05 kb using genomic DNA of the wild type strain and mutant M145E\textsubscript{1} as templates. For mutant of M145E\textsubscript{2}, the amplified DNA fragments are 2.25 kb and 2.73 kb using genomic DNA of the wild type strain and mutant M145E\textsubscript{2} as templates (Fig. 3). The results of confirmed PCR indicated that the mutants were constructed successfully. Furthermore, the results of southern blotting are shown in Figure 4. The mutant M145E\textsubscript{1} gave rise to 2.8 kb and 2.1 kb bands and M145E\textsubscript{2} gave rise to 3.9 kb and 1.5 kb bands, while wild-type strain yielded a 4.9 kb band as control. The obvious difference between the mutants and wild-type strain confirmed that the kanamycin resistance gene had inserted and disrupted the target genes of \textit{ecrE\textsubscript{1}} and \textit{ecrE\textsubscript{2}} in chromosomal DNA, respectively.
Fig. 3. Confirmation of mutants using PCR. (A) Illustration of PCR reaction; (B) 3, product from M145E1 (3.05 kb); 3C, product from wild-type strain genome (control, 3.15 kb); 4, product from M145E2 (2.73 kb); 4C, product from wild-type strain genome (control, 2.25 kb).

Fig. 4. Southern blotting analysis of mutants. (A) Southern blotting. The genome DNA was digested with PvuII. Two hybrid bands of mutant M145E1 were 2.1 kb and 2.8 kb. For mutant M145E2, 3.9 kb and 1.5 kb bands were detected; only 4.9 kb hybrid band presented in wild-type strain M145. (B) Illustration of the predicted hybrid fragments.

Fig. 5. Morphology of the mutants and wild-type strain. The colony images were taken after incubated on R5 plate for 9 days. (A) wild-type, (B) M145E1, (C) M145E2.

Fig. 6. Growth curves of mutants and wild-type strain.

Morphological investigation
As shown in Figure 5, the colony images of mutants and wild-type strain M145 showed that there was no difference in morphology between mutant and the wild type strain colony suggesting that they did not involve in the morphological differentiation. But the color of the mutant strains indicated that Red antibiotic production decreased. This result was indicated by the fermentation analysis, too.

Growth profile and antibiotics assay of mutants
Disruption of ecrE1/ecrE2 influenced the cell growth and biosynthesis of antibiotics (Li et al. 2004). The growth profiles and production of antibiotics of M145E1, M145E2 and wild-type M145 were therefore investigated. The disruption of ecrE1 and ecrE2 did not alter the growth process dramatically (Fig. 6). Act production of M145E1 and M145E2 almost did not give rise to the decrease compared with that of the wild-type strain (Fig. 7A). For antibiotic Red, the mutants M145E1 and M145E2 exhibited obvious phenomena of low yield compared with that of the wild-type strain. The decrease scope of production was about 40% (Fig. 7B). The results reflected that the ecrE1/ecrE2 genes markedly affected the production of antibiotic Red rather than Act, suggesting that ecrE1/ecrE2 might belong to a pair of
The transcription level of the red gene cluster was investigated during the culture progress of mutants and wild-type cells. The transcription levels of redD, redZ, and actII-ORF4 were investigated during the culture progress of mutants and wild-type cells. The results are shown in Figure 8. From 15 h to 70 h, the transcription levels of both redD and redZ in mutant strains compared with wild-type cells showed distinct decrease. However, the transcription level of actII-ORF4 in mutant strains and wild-type cells had no significant change. At 30 h, the transcription difference reached the most followed with finishing the phenomena. The result indicated that the two-component system ecrE1/ecrE2 played a role in the biosynthesis of red through coordination with the transcription of redD and redZ during the cell growth.

Discussion

Bioinformatics analysis indicated that the amino acid sequence identity between EcrA1 and EcrE2 and between Ecra2 and Ecra1 is 33.33% and 56.46%, respectively. The result implied that ecrE2/ecrE1 and ecra1/ecra2 might play the similar role in regulating antibiotics biosynthesis. Based on the significant regulation of ecrA1/ecra2 on Red production (Li et al. 2004), the ecrE1/ecrE2 was assumed to have a similar function.

Previous studies indicated that some specific genes could trigger or inhibit the transcription of the two pathway-specific regulatory genes actII-ORF4 and redD, which would accordingly give rise to the production alteration of antibiotics Act and Red, respectively (Takano et al. 1992; Arias et al. 1999). TCSs can subtly regulate antibiotic biosynthesis in S. coelicolor and most of these regulatory elements have a negative function such as absA1/absA2, afsQ1/afsQ2 and cutR/cutS (Ishizuka et al. 1992; Chang et al. 1996; Aceti & Champness 1998; Anderson et al. 2001). However, ecrE1/ecrE2 genes positively regulated the Red biosynthesis in our study. More interestingly, ecrE1/ecrE2 markedly affected Red production, but not Act production, which demonstrated that the ecrE1/ecrE2 genes may be involved in the synthesis of Red similar to that of ecrA1/ecra2. Furthermore, the regulatory ability of ecrA1/ecra2 is stronger than that of ecrE1/ecrE2 probably due to the minute differences in structure between EcrE2 and Ecra1, which both encode the response regulators. It is thus shown the ecrE1/ecrE2 and ecrA1/ecra2 might belong to one family in the TCSs positively regulating Red synthesis. In addition, the ecrE1/ecrE2 genes disruption did not influence the colony morphology, suggesting that they are not involved in the morphological differentiation.

In some studies, if the transcription of the two pathway-specific regulatory genes actII-ORF4 and redD were triggered or inhibited by some specific genes, the corresponding production of antibiotics Act and Red changed in the same manner (Takano et al. 1992; Arias et al. 1999). Transcription analysis demonstrated...
that the transcription levels of redD and redZ in mutant strains have slightly decreased compared with S. coelicolor M145, which suggested that may regulate the biosynthesis of Red through influencing the transcription of redD and redZ.

Since regulatory network about antibiotic biosynthesis is very complicated, a thorough analysis of their interaction relationship is essential for a complete understanding of gene regulatory pattern and morphology, including physiological development in Streptomyces and other complex microorganisms. The result of our investigation is elementary for proving the role of the ecrE1/ecrE2 in regulating the antibiotic synthesis of S. coelicolor. The TCSs ecrE1/ecrE2 and ecrA1/ecrA2 may play a positive regulating role in Red synthesis, which is a new insight into TCSs regulating antibiotic production in S. coelicolor M145.

Acknowledgements

This work was supported by grants from the China Scholarship Council. We would like to express our heartfelt thanks to Dr. David H. Sherman in the University of Michigan for providing pKC1139 and E. coli ET12567, and we would also like to thank Dr. Paul Kretchmer (kretchmer@sfedit.net) at San Francisco for his editing this manuscript.

References


